

# Identification of *Chlamydia pneumoniae* Proteins in the Transition from Reticulate to Elementary Body Formation\*

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*Chlamydia pneumoniae* is an important human respiratory pathogen that is responsible for an estimated 10% of community-acquired pneumonia and 5% of bronchitis and sinusitis cases. We examined changes in global protein expression profiles associated with the redifferentiation of reticulate body (RB) to elementary body (EB) as *C. pneumoniae* cells progressed from 24 to 48 h postinfection in HEp2 cells. Proteins corresponding to those showing the greatest changes in abundance in the beginning of the RB to EB transition were then identified from purified EBs. Among the 300 spots recognized, 35 proteins that were expressed at sufficiently high levels were identified by mass spectrometry. We identified *C. pneumoniae* proteins that showed more than 2-fold increases in abundance in the early stages of RB to EB transition, including several associated with amino acid and cofactor biosynthesis (Ndk, TrxA, Adk, PyrH, and BirA), maintenance of cytoplasmic protein function (GroEL/ES, DnaK, DksA, GrpE, HtrA, ClpP, ClpB, and Map), modification of the bacterial cell surface (CrpA, OmpA, and OmcB), energy metabolism (Tal and Pyk), and the putative transcriptional regulator TctD. This study identified *C. pneumoniae* proteins involved in the process of redifferentiation into mature, infective EBs and indicates bacterial metabolic pathways that may be involved in this transition. The proteins involved in RB to EB transition are key to *C. pneumoniae* infection and are perhaps suitable targets for therapeutic intervention. *Molecular & Cellular Proteomics* 5: 2311–2318, 2006.

*Chlamydia pneumoniae* causes both acute and chronic human respiratory disease (1) and has been associated with the chronic inflammatory process important in atherosclerosis (2–4). This Gram-negative obligate intracellular pathogen has a distinct biphasic developmental cycle that is unique to *Chlamydia*. Infections initiate when the extracellular infectious

elementary body (EB)<sup>1</sup> enters host epithelial cells by endocytosis. In the intracellular vacuole formed, or inclusion, the EB differentiates into a metabolically active reticulate body (RB) over the first 9 h and begins to replicate by binary fission by 19 h (5). RBs subsequently begin to redifferentiate into EBs, which are first detectable at 48 h and released by host cell lysis at 60–84 h postinfection (hpi) (5, 6). Formation and release of EBs is necessary to initiate new cycles of infection.

Although the temporal aspects and morphological progress of the *C. pneumoniae* developmental cycle have been characterized by microscopy (5, 6), the associated changes in cellular composition, metabolic activities, and regulatory mechanisms constituting this infection cycle remain largely unknown. Information from the *C. pneumoniae* genome sequence (7, 8) and studies of *C. trachomatis* using whole-genome gene expression profiling (9, 10) indicate that *C. pneumoniae* likely also relies on coordinated regulation of a specific set of genes to achieve its efficient biphasic infection cycle. Defining these stages and the associated microbial factors is now possible using powerful new genetic and biochemical analysis methods.

To better understand and identify microbial factors associated with the initial stages of *C. pneumoniae* transition from an intracellular replicating RB into an infectious EB under normal growth conditions, we used a global protein analysis approach based on two-dimensional electrophoresis, computer image analysis, and identification of bacterial proteins by mass spectrometry. We were able to detect substantial reproducible differences in protein expression profiles from 24 to 48 hpi and to identify 35 differentially expressed proteins that could be obtained in sufficient quantities from fully differentiated EBs. The majority of these proteins are predicted to be involved in translation and protein metabolism from studies of other bacterial species. Others included a potentially important putative transcriptional regulator and several that are likely associated with cell wall remodeling that occurs during differentiation.

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<sup>1</sup> The abbreviations used are: EB, elementary body; RB, reticulate body; hpi, hours postinfection; HEp2, human epithelial type 2; TEM, transmission electron microscope; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; PPP, pentose phosphate pathway.

## EXPERIMENTAL PROCEDURES

**Cell Cultures**—HEp2 cells (ATCC CCL-23) were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in Iscove's maintenance medium (Cellgro, Washington, D. C.) as described previously (11). Cells were grown in 75-cm<sup>2</sup> flasks (Costar, Cambridge, MA) at 37 °C in 5% CO<sub>2</sub> for 24 h to achieve confluency of the monolayer and harvested with trypsin-EDTA.

**Bacterial Cultures**—*C. pneumoniae* A-03 (ATCC VR-1452) was previously isolated from a coronary atheroma of a patient undergoing heart transplantation surgery at the Jewish Hospital Heart and Lung Institute, Louisville, KY (3). *C. pneumoniae* were propagated in HEp2 cell monolayers in Iscove's growth medium. EBs were harvested at 72 h and purified by disruption of HEp2 cell monolayers with a cell scraper, sonication, and centrifugation over a Renografin density gradient as described previously (11). EB suspensions in sucrose-phosphate-glutamic acid buffer were stored at −80 °C.

**Cell Culture Infections**—HEp-2 cells were seeded in 6-well plates at  $0.5 \times 10^6$  cells/well in Iscove's maintenance medium and incubated in 5% CO<sub>2</sub> at 37 °C overnight. Cells were subsequently inoculated with  $0.5 \times 10^8$  inclusion-forming units/well (multiplicity of infection, 100:1) of *C. pneumoniae* in 2 ml of Iscove's growth medium, centrifuged at  $675 \times g$  for 1 h at 10 °C, and incubated at 37 °C in 5% CO<sub>2</sub> for 24 h.

**Transmission Electron Microscope (TEM) Analysis**—TEM was used to visualize *C. pneumoniae* interactions with host cells by analyzing the cell cultures as follows. Specimens were fixed with 3% glutaraldehyde (Sigma) fixative in 0.1 M phosphate buffer at pH 7.4. After fixation for 1 h at room temperature, cells were scraped off, washed in 0.1 M phosphate buffer, postfixed in 1% osmium tetroxide, and embedded in Spurr epoxy resin according to standard procedures (12). Ultrathin sections (50–100 nm) were cut and stained with uranyl acetate and lead citrate stains and examined with a JEOL 1200EX transmission electron microscope.

**Protein Labeling**—For global analysis of bacterial proteins, infected HEp-2 cells were pulse-labeled for 2 h (at 22 and 46 hpi, respectively) in methionine/cysteine-free RPMI 1640 medium (Cellgro, Herndon, VA) containing 100  $\mu$ Ci of [<sup>35</sup>S]methionine/cysteine (Redivue Pro-Mix)/ml (Amersham Biosciences) and 500  $\mu$ g of cycloheximide/ml. At the end of the 2-h labeling period, HEp-2 cells were washed in cold phosphate-buffered saline, scraped with a cell scraper, and pelleted by centrifugation at  $16,000 \times g$ .

**Protein Extraction**—*C. pneumoniae*-infected HEp-2 cell pellets were resuspended in 30  $\mu$ l of buffer containing 2% (w/v) Sarkosyl, 1% (w/v) octyl  $\beta$ -D-1-thioglucoopyranoside, 2 mM tributylphosphine, and 5  $\mu$ l of protease inhibitors (leupeptin and aprotinin). Samples were sonicated (cell dismembrator, Model 100, Fisher Scientific) for 5 s, boiled for 5 min, and then cooled to room temperature. One hundred microliters of thiourea lysis buffer (7 M urea, 2 M thiourea, 10 mM Tris, 2 mM tributylphosphine, and 2% (v/v) Ampholine) were added followed by the addition of 3.0  $\mu$ l of a mixture of 50 mM MgCl<sub>2</sub>, 476 mM Tris-HCl, 24 mM Tris base, 1.0 mg of DNase-1/ml, and 0.250 mg of RNase A/ml (pH 8.0); kept on ice for 10 min; and stored at −80 °C.

**Two-dimensional Gel Electrophoresis**—For generating electrophoretic protein maps of purified *C. pneumoniae* EB suspension, 750  $\mu$ g of protein extracts were first mixed with an IPG rehydration buffer as mentioned previously (13). Briefly for intracellular *C. pneumoniae* protein expression studies, 75  $\mu$ g of <sup>35</sup>S-labeled chlamydial proteins were mixed in IPG rehydration buffer and loaded onto IPG strips (Bio-Rad ReadyStrip™ IPG strip; strip length, 17 cm), allowed to rehydrate overnight, and isoelectrofocussed in a PROTEAN® IEF cell (Bio-Rad). A rapid advance voltage ramping method was applied as mentioned previously (13). After focusing was completed, IPG strips were equilibrated in equilibration base buffer with 2% (w/v) dithiothreitol followed by another equilibration step with 2.5% (w/v) iodoacetamide. The second dimension was carried out with polyacrylamide

gel electrophoresis (10% polyacrylamide formulated in Tris-HCl) on 19.3 cm  $\times$  18.3 cm  $\times$  1.0 mm slab gels (PROTEAN II precast ready gel) in a PROTEAN II xi multicell system (Bio-Rad) at 4 °C for 3.5 h under a 500-V maximum voltage with 1 $\times$  Tris-Tricine-SDS buffer used as the upper electrode buffer and 1 $\times$  Tris-glycine-SDS used as lower electrode buffer (Bio-Rad). Gels containing purified EB proteins were silver-stained, whereas gels containing radiolabeled chlamydial proteins were fixed in a solution containing 40% ethanol and 7.5% acetic acid for 30 min and treated with Amplify fluorographic reagent (Amersham Biosciences) for 30 min, vacuum-dried, and exposed to high density phosphorimaging screens (Bio-Rad Inc.) for 2.5 days. Images were scanned in a Molecular Imager® FX Pro Plus™ system (Bio-Rad).

**Image Analysis**—Protein spots were analyzed and quantified for differential expression patterns using PDQuest™ software version 7.1 (Bio-Rad), which interfaced directly with the PhosphorImager using ProteomeWorks software. Protein spots on different gels were aligned by the pattern recognition feature of the software. PDQuest software was also used for quantification in which pixel values for each identified spot were first normalized as a percentage of total pixel quantity of all valid spots for each individual gel image. After normalization, analytical tools in PDQuest software were used for statistical analysis of differential expression patterns.

**Identification of Protein Spots**—Spots of interest from the <sup>35</sup>S-labeled gels were then matched with images of up to 10-silver stained *C. pneumoniae* 72-h EB gels with PDQuest, and corresponding spots were excised from the silver-stained gels and treated with 20  $\mu$ g of trypsin/ml in 50 mM ammonium bicarbonate at 37 °C overnight. For those proteins that appeared in "tracts," multiple samples were collected to ensure they corresponded to a single protein. Two microliters of supernatant were mixed in an equal volume of saturated  $\alpha$ -cyano-4-hydroxycinnamic acid, 50% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid, and 0.8  $\mu$ l of the resulting solution was applied to the MALDI-MS template. Masses of peptide fragments were determined by MALDI-TOF analysis with a Micromass mass spectrometer. Patterns of measured masses were matched against theoretical masses and pI values of proteins found in the annotated National Center for Biotechnology (NCBI), Swiss-Prot, and TrEMBL databases accessible in the Expasy Molecular Biology server (expasy.cbr.nrc.ca/). Searches were performed with ProFound-Peptide Mapping software (The Rockefeller University Edition version 4.10.5) with restrictions to proteins from 1 to 100 kDa and mass tolerances of 100–150 ppm. Partial enzymatic cleavages leaving one cleavage site, oxidation of methionine, and modification of cysteine with iodoacetamide were considered in these searches. Sequence coverage >45% (p value <0.05) was considered as a positive match.

**Quantitative PCR**—RNA was isolated using TRIzol (Invitrogen) from 24- and 48-hpi *C. pneumoniae*-infected HEp-2 monolayers that were cultured in duplicate according to the method described by Hogan *et al.* (14). Contaminating DNA was removed by incubation with 40 units of RNase-free DNase and RNase inhibitor (Roche Applied Science) at 37 °C for 1 h followed by precipitation with ammonium acetate and ethanol and several 70% ethanol washes. The RNA was resuspended in 50  $\mu$ l of diethyl pyrocarbonate-treated double distilled H<sub>2</sub>O. The absence of genomic DNA was confirmed by 16 S PCR as described previously (15).

cDNA was generated from 1  $\mu$ g of total RNA as described previously (14) except that the random hexamers (1  $\mu$ g) were incubated with the RNA at 65 °C for 10 min before the addition of 12 units of RNase inhibitor and 50 units of Expand reverse transcriptase (Roche Applied Science) in 1 $\times$  Expand buffer in a 20- $\mu$ l reaction. The reaction mixture was incubated at 30 °C for 10 min followed by 42 °C for 45 min. Residual RNA was removed, and cDNA was purified as described previously (14) before storage at −20 °C.



TABLE I  
Primers for individual genes used in this study

*C. pneumoniae* CWL029 sequence (GenBank™ accession number AE001363) was used to design the primers.

Gene	5'end	Forward Primer	5'end	Reverse Primer
16S rRNA	494	5' GGGTAAAGAAGCACCGGCTAA	556	5' AACGCTAGCACCCCTCCGTATT
<i>ndk</i>	160	5' GAGCGTCCTTTTTTCCAAGAAC	236	5' TTTGCCCCTTCCAATACTAAACT
<i>trxA</i>	54	5' CGTTCTCGTTGATTTCTTTGCA	129	5' CGCAGCAAGATTTTCTAAGATAGGA
<i>adk</i>	228	5' GCAAAGCCAAGCCTGCTCTA	297	5' AAGATGCGCCTGATCTAAGGTT
<i>pyrH</i>	153	5' CGGCGGTGGCAATATTTTAA	226	5' TTTGATCTGCCGATACACGATT
<i>birA</i>	478	5' AAGAAGCTCCCTGGTAGTCTCAAG	445	5' TGTCGGACAACCTGCAACAT
<i>crpA</i>	95	5' GGAGTGTACCCGCTGGTCTAC	174	5' TTTTGTCTTTGCCATCCTATGA
<i>ompA</i>	4	5' AAAAAACTCTTAAAGTCGGCGTTATT	69	5' GGCTTGTAAGGAGCCAACAGA
<i>omcB</i>	458	5' CTGAATTCGTAAGCAGTGATCCA	530	5' AGGCGATCGATTTTCCAGACT
<i>ahpC</i>	4	5' ACACTCTCCCTAGTTGGAAGGAA	71	5' CACGTTTCGCCATTAACAACA
<i>gspD</i>	1065	5' GAACCCTACGGATAAAACAGTATTTTG	1139	5' GAAAGCAATGCCGCCAACT
<i>tal</i>	125	5' TGGCCCAAGAACCCAAATT	194	5' TCACCGTTCTGTCCGATTCC
<i>pyk</i>	1056	5' TCTTCAGGTGTCCCCCTATCTCT	1106	5' TGGCGTCTGCCCTTTCTG
<i>groEL_1</i>	291	5' AGCAATCTATAGCGAAGGTCTAAGAAA	370	5' CGGCTTTGTGATACCTCTTTT
<i>groES</i>	15	5' AACGACCCTCCGAATTAACC	89	5' CCACGAGCAGTGGCTTCTTC
<i>dnaK</i>	484	5' CGCATTGCAGGTCTAGATGTAAA	558	5' TTTATCGATTCCGTAGGCAAGAG
<i>dksA</i>	244	5' GAGTCTTCTTACGGGATTTGTGATG	317	5' GCATAGGGAATGGCTATCAACCT
<i>grpE</i>	369	5' TTCTTCAATAGGCCAAAAGTTTAACC	447	5' CCCCTCAGGAACCTCAGAAGTCT
<i>htrA</i>	177	5' TCCCGCTGTTGTGTACATAGAAA	248	5' CACGGCGTCCAGGAGAAG
<i>clpP_1</i>	236	5' CACCCGCTCACTACTGTTGTGACA	308	5' CCAGGAGCTGCACATAAACTCA
<i>clpB</i>	1710	5' GGTAGGACAGCCTTTTGCAGTCT	1789	5' GACGTTGAGGATCATTTAAACCTACA
<i>map</i>	163	5' GCCTGCCAAATTACAGCAAGA	246	5' GAGCTCATCCAACCTATTTGTAGTG
<i>greA</i>	534	5' TTTTCGAGACGGCATTTAAAGTT	600	5' TCCAAACCGTCGTGAAAAG
<i>rpoA</i>	382	5' GCCGCAGCTAATGGACAAA	461	5' TGATCTGGGTAAACGGCTTCA
<i>tctD</i>	258	5' TCAAGGAGCAACAGGGTATCTTC	335	5' TGGCGTAGGAAAGCTCTGATC
<i>tsf</i>	172	5' AAAGAAGGCATCATAGCAGCTAAAA	247	5' CAAATCTGTCTCTACGTTCACTTCAA
<i>tufA</i>	662	5' TCTCTGGTCTGTTGTTGTTGTT	741	5' TCCCACGAGCTGAACCTTATCA
<i>efp1</i>	81	5' GGTGGCAGGGCCCAA	159	5' GGATTCTGATGTTGTTATTGAGAGAAAT
<i>pfrA</i>	802	5' GATAAGGCCATGCGGATTCTT	870	5' CGCCTCGTTATGGCGTTTT
<i>rp1L/r17</i>	104	5' CTGCTTCTGCTCCCGTAGTTG	177	5' TTCTGTAGGTTCCGGCAGCAA
<i>rpsA/rs1</i>	730	5' GGCTTCTCCACATTACCGATATGA	806	5' AACTCTTGATTCAATTCGACCATT
Cpn0710	154	5' CGTATGCAGATCTTATCACAGTATATGGA	233	5' GCCATTGTGATCATCTCTGTGTTT
Cpn0518	746	5' CAGAAAAATTTGATACGGAAGAAGAA	827	5' TTGGTAAGATTACAGCTGGTCTTCA
Cpn0742	292	5' GATGAGTATCGAAAAGAGACAGAAAATTT	394	5' TGGTAGATGACAAATTTTGTGTTTG
Cpn1032	256	5' CGTGGTGCTGCTCTCTCTGA	332	5' CCATTTTATCTTTACCCAGCAA
Cpn0216	115	5' TCCATTTTATCTGTCAATTTGTTTG	196	5' AGCAGCCGAGTCCTAGAGTAAAAAG
Cpn0917	293	5' TTGCTGAAGTTCGTGGTGACA	368	5' CCTTCTTGCAAAGATAACCATTGA

Primers (Table I) for quantitative PCR were designed for the 35 genes for proteins identified as differentially expressed (Primer-Express 2.0, Applied Biosystems, Melbourne, Australia). Standard controls for each primer pair were generated by amplifying *C. pneumoniae* genomic DNA followed by purification via QIAquick columns (Qiagen, Victoria, Australia). The cDNA was quantitated using the ABI Prism 7000 (Applied Biosystems) in a standard reaction containing 1.0  $\mu$ l of cDNA in a final concentration of 1 $\times$  ABI SYBR green Master Mix (Applied Biosystems) and a 10  $\mu$ M concentration of each primer (forward and reverse, Table I) in a final volume of 25  $\mu$ l. Cycling parameters for all reactions were: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 1 min of annealing and extension at 60 °C

with melting curve analysis from 60 to 95 °C with data acquisition every 1 °C. Standard curves were generated for each gene using 10<sup>3</sup>, 10<sup>5</sup>, 10<sup>7</sup>, and 10<sup>9</sup> copies of each standard, and negative controls were performed for each gene. All reactions were performed in triplicate using the standard reaction mixture and cycling conditions as described above. ABI Prism 7000 SDS Software version 1.0 (Applied Biosystems) was used to analyze amplification curves for each cDNA and determine the copy number. Grubb's test was used to identify outlying copy numbers that were subsequently excluded from further calculations. Data are presented as the mean cDNA copy number obtained for each gene divided by the corresponding mean 16 S cDNA values.

FIG. 1. Transmission electron micrograph indicating morphological features of *C. pneumoniae* grown in HEp2 cells showing typical RBs and an absence of EBs at 24 hpi (A) and typical EBs and RBs at 48 hpi (B), confirming asynchronous developmental cycle due to redifferentiation of RBs to EBs.

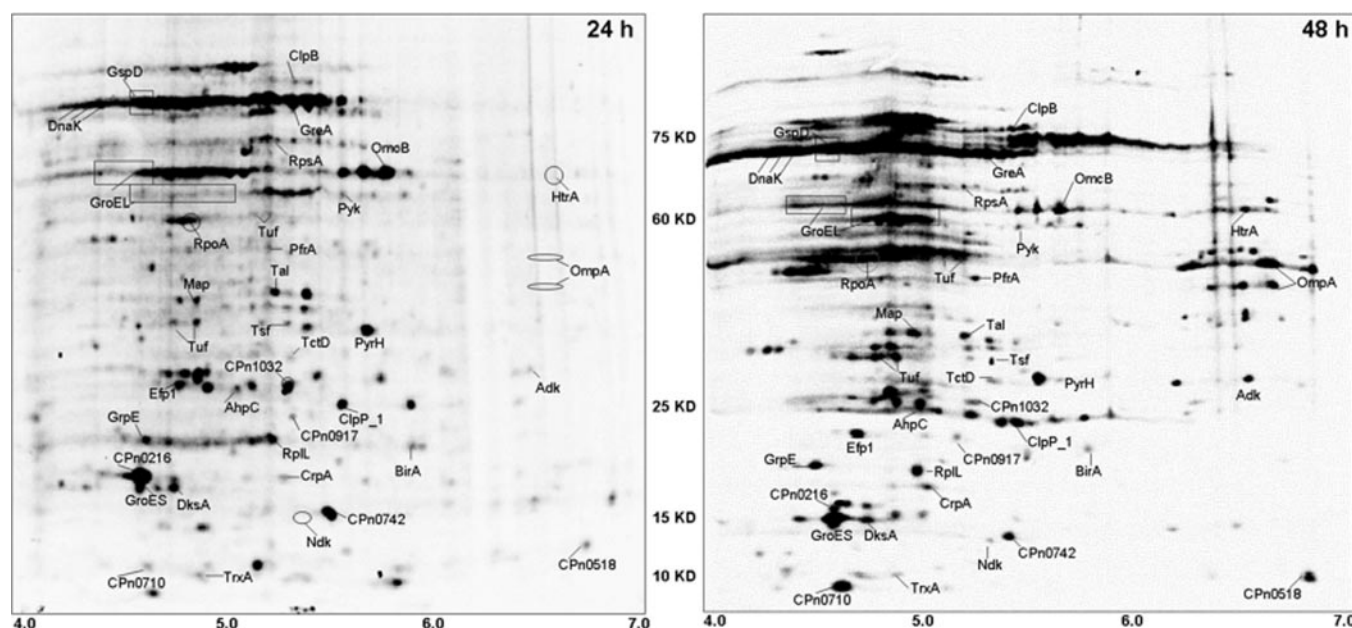
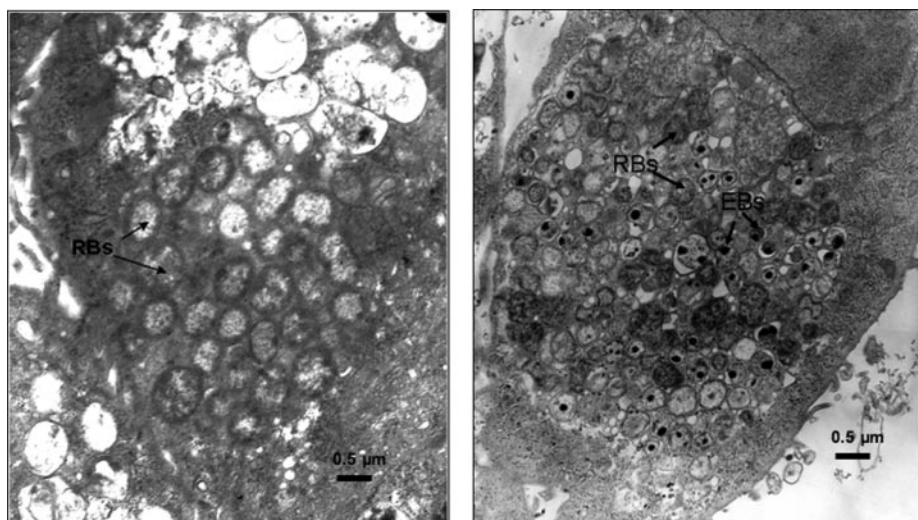


FIG. 2. Two-dimensional electrophoretic map of *C. pneumoniae* proteins expressed at 24 and 48 hpi in HEp2 cells. Representative gels of 10 experiments are shown. A summary of the -fold changes is listed in Tables II and III.

## RESULTS

**Ultrastructural Analysis of *C. pneumoniae* Development**—Similar to that which has been described previously for HeLa cells (5), *C. pneumoniae* EBs were internalized by HEp2 cells and by 24 h showed complete differentiation into larger replicating RBs by TEM examination (Fig. 1A). RBs were observed throughout the entire inclusion. By 48 h postinfection, the transition to EBs had begun (Fig. 1B) with ~50% of *C. pneumoniae* showing characteristic reduced size and condensed nucleoid structure. We could also identify intermediate transitional forms as cell differentiation had become asynchronous by 48 h. We therefore chose to compare protein expression at 24 and 48 h to identify those bacterial proteins

that contribute in the initial stages of the developmental transition from RB to infectious EBs.

**Temporal Analysis of *C. pneumoniae* Protein Expression in Infected HEp2 Cells**—To examine expression of chlamydial proteins at 24 and 48 h postinfection, infected HEp2 cells were pulse-labeled for 2 h with [<sup>35</sup>S]methionine/cysteine in the presence of cycloheximide at either 22 or 46 h. Total protein extracts were prepared from lysed monolayers, and the radiolabeled bacterial proteins were analyzed by two-dimensional gel electrophoresis. Fig. 2 shows two representative PhosphorImager scans of gels showing proteins resolved among those expressed by *C. pneumoniae* at 24 and 48 h. More than 300 protein spots could be detected in pH 3–11



TABLE II  
Expression profiles of *C. pneumoniae* proteins at 48 versus 24 hpi

The differential regulation pattern at 48 hpi was compared with respect to 24 hpi. The 24-hpi sample was considered as 1-fold. More than 2.0-fold change was considered significant. Proteins have been grouped together according to functional assignments provided at [www.gram.au.dk/Cpn\\_table\\_link.html](http://www.gram.au.dk/Cpn_table_link.html). OMP, outer membrane protein.

Functional assignments	Function	Expression profile
Biosynthesis of amino acids and cofactors		
Ndk	Nucleoside-2-phosphate kinase	Present (up) <sup>a</sup>
TrxA	Thioredoxin	4.5 (down)
Adk	Adenylate kinase	14.8 (up)
PyrH	UMP kinase	2.1 (up)
BirA	Biotin synthase	Present (up) <sup>a</sup>
Cell envelope		
CrpA	15-kDa cystine-rich protein	6.5 (up)
OmpA	Outer membrane protein	Present (up) <sup>a</sup>
OmcB	Cystine-rich OMP	3.3 (up)
Cellular processes		
AhpC	Alkyl hydroperoxide reductase	6.2 (up)
GspD	General secretion protein D	4.6 (down)
Energy metabolism		
Tal	Transaldolase	5.4 (up)
Pyk	Pyruvate kinase	4.9 (up)
Protein folding, assembly, and modification		
GroEL_1	Heat shock protein-60	6.4 (up)
GroES	Heat shock protein-10	3.3 (up)
DnaK	Heat shock protein-70	5.0 (up)
DksA	DnaK suppressor	1.9 (up)
GrpE	HSP-70 cofactor	3.1 (up)
HtrA	DO serine protease	14.7 (up)
ClpP_1	Endopeptidase Clp1	5.2 (up)
ClpB	Clp protease ATPase	11.6 (up)
Transcription		
GreA	Transcription elongation factor	9.3 (up)
RpoA	RNA polymerase $\alpha$ chain	7.3 (up)
TctD	Transcription regulatory protein	5.6 (up)
Translation		
Map	Methionine aminopeptidase	8.7 (up)
Tsf	Elongation factor TS	9.0 (up)
TufA	Elongation factor TU	7.1 (up)
Efp1	Elongation factor P1	2.9 (up)
PfrA	Peptide chain release factor-1	4.8 (up)
RplL (R17)	50 S ribosomal protein L7/L12	4.5 (up)
RpsA (Rs1)	30 S ribosomal protein S1	2.8 (up)
Hypothetical proteins		
Cpn0710		8.6 (up)
CPn0518		12.8 (up)
CPn0742		2.8 (down)
CPn0216		2.2 (down)
CPn0917	Hydrolase/phosphatase homologue	5.0 (up)

<sup>a</sup> Present in 48 hpi only,  $\geq 10$ -fold.

range with a combination of IPG strips pl 4–7, 6–11, and 3–10. Gels were then matched with 6–10 independent silver-stained gels that were used to separate larger amounts of unlabeled proteins from purified EB preparations. This procedure was necessary to obtain sufficient material for protein identification by MALDI-MS. Each mapped protein spot (Fig. 2) was quantified in terms of a percentage of the sum of all radioactive spots detected on the same gel to allow comparison of gels showing differences in overall image intensity. A

subset of those proteins that showed the greatest differential expression at 24 and 48 h for which we were able to obtain sufficient protein sample from silver-stained gels ( $n = 35$ ) were then characterized by MALDI-TOF-MS. A summary of these analyses is presented in Table II.

We considered a threshold of 2-fold as a significant change in protein level based on previous studies (9, 16, 17). Thirty-one of the 35 proteins (89%) identified to be temporally correlated with the initial steps in the morphological redifferenti-

ation from RBs into infectious EBs showed increased expression, whereas only four proteins (11%) identified were found to decrease with the onset of bacterial redifferentiation (Table II). We likely identified more proteins whose expression increased as we were only able to identify proteins whose expression remained at sufficient levels for MALDI-TOF-MS analysis in purified EBs prepared at a later time point (see “Experimental Procedures”). Among the down-regulated proteins, thioredoxin (TrxA) and general secretion protein D (GspD) were down-regulated by 4.5- and 4.6-fold, respectively (Table II). Levels of *C. pneumoniae* hypothetical proteins CPn0742 and Cpn0216 decreased 2.8- and 2.2-fold, respectively.

Proteins whose levels showed relatively small increases (up to 4-fold) as bacteria began differentiation into EBs included uridine monophosphate kinase (PyrH), the cysteine-rich outer membrane protein OmcB, heat shock protein-10 (GroES), the DnaK suppressor DksA, heat shock protein-70 cofactor (GrpE), translation elongation factor P1 (Efp1), and 30 S ribosomal protein S1 (RpsA/Rs1) (Table II). Proteins showing higher levels of increase (4–6-fold) included transaldolase (Tal), pyruvate kinase (Pyk), heat shock protein-70 (DnaK), endopeptidase Clp 1 (ClpP\_1), helix-turn-helix transcriptional regulatory protein (TctD), peptide chain release factor-1 (PfrA), 50 S ribosomal protein L7/L12 (RplL/RI7), and CPn0917 (hydrolase/phosphatase homologue). Seven proteins showed larger relative increases (6–10-fold); a 15-kDa cysteine-rich protein (CrpA), alkyl hydroperoxide reductase (AhpC), heat shock protein 60 kDa (GroEL), methionine aminopeptidase (Map), transcription elongation factor (GreA), RNA polymerase  $\alpha$  chain (RpoA), elongation factor TS (Tsf), elongation factor TU (TufA), and hypothetical protein CPn0710. Four proteins were found to increase more than 10-fold: adenylate kinase (Adk), DO serine protease (HtrA), Clp protease (ClpB), and hypothetical protein CPn0518. Ndk, BirA, and OmpA showed the most dramatic differential expression with detection only at 48 h.

**Temporal Analysis of *C. pneumoniae* Gene Expression in Infected HEp2 Cells**—Quantitative fluorescence-monitored RT-PCR was performed for the 35 mRNAs encoding EB proteins identified by MALDI-TOF-MS. 16 S rRNA was used as an internal standard for comparison of transcript levels at 24 and 48 h as described previously for other chlamydial species (9, 14). These data are summarized in Table III. Only 10 RNAs showed significant changes (>2-fold) in steady state levels at 48 h: *crpA* (12.4-fold up), *dksA* (3.2-fold up), CPn0216 (2.7-fold up), CPn0710 (5.1-fold up), *efp1* (3-fold down), *tctD* (21.6-fold up), *omcB* (6.6-fold up), *ompA* (2.4-fold up), *clpB* (7.7-fold up), and CPn0518 (2.8-fold down). The remaining 25 mRNAs encoding proteins whose levels increased during this initial stage of morphological differentiation remained unchanged between 24 and 48 h in these assays. Levels of three transcripts, *efp1*, CPn0518, and CPn0216, did not correspond to the respective protein levels. It is likely that the level of post-

TABLE III  
Differential expression levels of mRNA compared with protein levels in *C. pneumoniae*

Functional assignments	mRNA expression profile	Protein expression profile
Cell envelope		
<i>CrpA</i>	12.4 (up)	6.5 (up)
<i>ompA</i>	2.4 (up)	Present (up) <sup>a</sup>
<i>omcB</i>	6.6 (up)	3.3 (up)
Protein folding, assembly and modification		
<i>dksA</i>	3.2 (up)	1.9 (up)
<i>clpB</i>	7.7 (up)	11.6 (up)
Transcription		
<i>tctD</i>	21.6 (up)	5.6 (up)
Translation		
<i>Efp1</i>	3.0 (down)	2.9 (up)
Hypothetical proteins		
CPn0710	5.1 (up)	8.6 (up)
CPn0518	2.8 (down)	12.8 (up)
CPn0216	2.7 (up)	2.2 (down)

<sup>a</sup> Present in 48 hpi only,  $\geq 10$ -fold.

transcriptional and post-translational regulation of these genes plays an important role. However, we were also able to see obvious increases for a subset of mRNAs in these same assays (Table III).

DISCUSSION

The developmental cycle of *C. pneumoniae* in HEp2 cells is an asynchronous process, resulting in a heterogeneous population of bacterial morphologies at any given time point. In the experiments described here, we normalized individual protein expression to the entire population at the 24- and 48-h time points and determined quantitative changes in expression levels.

We identified 35 *C. pneumoniae* proteins from nine functional groups, representing 11.6% of the total proteins expressed, whose expression levels changed as bacteria began the secondary differentiation from intracellular replicating RBs into infectious EBs. Almost one-half of these were bacterial factors associated with protein metabolism, including proteases, chaperones, and translation factors. Alterations of ribosome function and cell protein remodeling and turnover were a central recognizable aspect of the onset of this morphological differentiation. Our analysis of the mRNAs encoding these 35 proteins was also consistent with a model where post-transcriptional regulatory mechanisms may play a role in *C. pneumoniae* differentiation. Although we have not tested this hypothesis in this study, further studies will be important to determine these mechanisms and to define transcriptional regulation involving the TctD transcription factor.

The expression of three cell membrane proteins genes (*crpA*, *ompA*, and *omcB*) was found to increase at both the mRNA and protein level as bacteria progressed from 24 to 48 h postinfection. mRNAs encoding these proteins have also

been shown to increase in other *Chlamydia* species as EB formation begins (9, 10). OmcB is a cysteine-rich outer membrane protein that undergoes post-translational proteolytic processing (18) and forms a highly disulfide-cross-linked outer membrane complex with OmpA. CrpA is also a cysteine-rich protein whose cross-linking with OmpA, the major outer membrane protein, may contribute to relative environmental resistance of the EB.

A transaldolase (Tal) and pyruvate kinase (Pyk) were increased in expression as bacteria began redifferentiation into EBs during HEp2 infection. Both of these proteins have predicted roles in bacterial energy metabolism. Tal is a rate-limiting enzyme of the non-oxidative reaction in pentose phosphate pathway (PPP) (19). Together with transketolase, Tal provides a link between glycolysis and the PPP. Increased Tal expression during redifferentiation may allow increased catabolism of sugars, and the PPP also potentially provides the cell with intermediates for the synthesis of amino acids, nucleotides, vitamins, and cell wall constituents (19). Increases and some decreases in the levels of other proteins in biosynthetic pathways were also seen, indicating a realignment of metabolism as bacteria began the active process of EB formation. For example, the enzyme Pyk catalyzes the final step in glycolysis by converting phosphoenolpyruvate to pyruvate with concomitant phosphorylation of ADP to ATP (20). Both Pyk and Tal have previously been shown to increase during *C. trachomatis* replication, providing ATP for metabolism (21, 22).

A final important class of protein whose increased synthesis was seen upon differentiation into EB were specific to *Chlamydia* (Table II), including CPn0216, which shows no similarity to any protein in any other species. Similarly the large class of *Chlamydia trachomatis* genes consisting of unknown function whose expression was increased with EB formation are those lacking homologues in other species (10). These are likely components of the infectious EB that impart its unique characteristics, including the ability to attach to and enter a wide variety of host cells.

An important aspect of any natural population of bacteria is the heterogeneity within as described for the *C. pneumoniae* populations at 48 hpi. The presence of RBs, EBs, and transitional forms in this population prevents an entirely accurate numerical determination of the specific magnitude of differential gene expression and resulting RNA and protein levels in individual cells during redifferentiation, and we are only able to report an average level for all types of cells present in this population. The specific value given in Table II therefore describes the relative degree of increased expression and must be viewed as an estimate. This acknowledged difficulty did not prevent us from identifying a small core group of proteins whose levels do increase relative to all detectable cellular proteins in our assays. These bacterial factors are clearly important in the process by which *C. pneumoniae* begins redifferentiation back into infectious EBs.

Although specific morphological changes in the *Chlamydia* life cycle have been carefully described for several decades, our knowledge of the biochemical nature of this developmental process remains lacking. Identification of 35 proteins whose expression levels change as *C. pneumoniae* differentiates into infectious EBs late in infection contributes important initial basic information about this largely uncharacterized molecular process. Although with this report we have only begun to describe the participants in this process, we have identified what are likely to be significant proteins in the EB formation process that may reveal biomarkers for therapeutic strategies.

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#### REFERENCES

1. Kuo, C.-C., Jackson, L. A., Campbell, L. A., and Grayston, J. T. (1995) *Chlamydia pneumoniae* (TWAR). *Clin. Microbiol. Rev.* **8**, 451–461
2. Danesh, J., Collins, R., and Peto, R. (1997) Chronic infections and coronary heart disease: is there a link? *Lancet* **350**, 430–436
3. Ramirez, J. A., Ahkee, S., Summersgill, J. T., Ganzel, B. L., Ogden, L. L., Quinn, T. C., Gaydos, C. A., Bobo, L. L., Hammerschlag, M. R., Roblin, P. M., LeBar, W., Grayston, J. T., Kuo, C.-C., Campbell, L. A., Patton, D. L., Dean, D., and Schachter, J. (1996) Isolation of *Chlamydia pneumoniae* from the coronary artery of a patient with coronary atherosclerosis. *Ann. Intern. Med.* **125**, 979–982
4. Saikku, P., Leinonen, M., Mattila, K., Ekman, M. R., Nieminen, M. S., Makela, P. H., Huttunen, J. K., and Valtonen, V. (1988) Serological evidence of an association of a novel Chlamydia, TWAR, with chronic coronary heart disease and acute myocardial infarction. *Lancet* **2**, 983–986
5. Wolf, K., Fischer, E., and Hackstadt, T. (2000) Ultrastructural analysis of developmental events in *Chlamydia pneumoniae*-infected cells. *Infect. Immun.* **68**, 2379–2385
6. Hatch, T. (1999) *Chlamydia: Intracellular Biology, Pathogenesis, and Immunity*, pp. 29–67, American Society for Microbiology Press, Washington, D. C.
7. Kalman, S., Mitchell, W., Marathe, R., Lammel, C., Fan, J., Hyman, R. W., Olinger, L., Grimwood, J., Davis, R. W., and Stephens, R. S. (1999) Comparative genomes of *Chlamydia pneumoniae* and *C. trachomatis*. *Nat. Genet.* **21**, 385–389
8. Shirai, M., Hirakawa, H., Kimoto, M., Tabuchi, M., Kishi, F., Ouchi, K., Shiba, T., Ishii, K., Hattori, M., Kuhara, S., and Nakazawa, T. (2000) Comparison of whole genome sequences of *Chlamydia pneumoniae* J138 from Japan and CWL029 from U. S. A. *Nucleic Acids Res.* **28**, 2311–2314
9. Belland, R. J., Zhong, G., Crane, D. D., Hogan, D., Sturdevant, D., Sharma, J., Beatty, W. L., and Caldwell, H. D. (2003) Genomic transcriptional profiling of the developmental cycle of *Chlamydia trachomatis*. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 8478–8483
10. Nicholson, T. L., Olinger, L., Chong, K., Schoolnik, G., and Stephens, R. S. (2003) Global stage-specific gene regulation during the developmental cycle of *Chlamydia trachomatis*. *J. Bacteriol.* **185**, 3179–3189

11. Mukhopadhyay, S., Clark, A. P., Sullivan, E. D., Miller, R. D., and Summersgill, J. T. (2004) Detailed protocol for purification of *Chlamydia pneumoniae* elementary bodies. *J. Clin. Microbiol.* **42**, 3288–3290
12. Robards, A. W., and Wilson, A. J. (1993) *Procedures in Electron Microscopy*, section 5.1–5.6, John Wiley & Sons Ltd., Chichester, UK
13. Mukhopadhyay, S., Miller, R. D., Sullivan, E. D., Theodoropoulos, C., Mathews, S. A., Timms, P., and Summersgill, J. T. (2006) Protein expression profiles of *Chlamydia pneumoniae* in models of persistence versus those of heat shock stress response. *Infect. Immun.* **74**, 3853–3863
14. Hogan, R., Mathews, S. A., Kutlin, A., Hammerschlag, M. R., and Timms, P. (2003) Differential expression of genes encoding membrane proteins between acute and continuous *Chlamydia pneumoniae* infections. *Microb. Pathog.* **34**, 11–16
15. Mathews, S. A., George, C., Flegg, C., Stenzel, D., and Timms, P. (2001) Differential expression of *ompA*, *ompB*, *pyk*, *nlpD* and *Cpn0585* genes between normal and interferon- $\gamma$  treated cultures of *Chlamydia pneumoniae*. *Microb. Pathog.* **30**, 337–345
16. Mader, U., Homuth, G., Scharf, C., Buttner, K., Bode, R., and Hecker, M. (2002) Transcriptome and proteome analysis of *Bacillus subtilis* gene expression modulated by amino acid availability. *J. Bacteriol.* **184**, 4288–4295
17. Yoon, S. H., Han, M.-J., Lee, S. Y., Jeong, K. J., Yoo, J.-S (2003) Combined transcriptome and proteome analysis of *Escherichia coli* during high cell density culture. *Biotechnol. Bioeng.* **81**, 753–767
18. Batteiger, B. E., Newhall, W. J., and Jones, R. B. (1985) Differences in outer membrane proteins of the lymphogranuloma venereum and trachoma biovars of *Chlamydia trachomatis*. *Infect. Immun.* **50**, 488–494
19. Sprenger, G. A. (1995) Genetics of pentose-phosphate pathway enzymes of *Escherichia coli* K-12. *Arch. Microbiol.* **164**, 324–330
20. Fothergill-Gilmore, L. A., and Michels, P. A. (1993) Evolution of glycolysis. *Prog. Biophys. Mol. Biol.* **59**, 105–235
21. Gerard, H. C., Freise, J., Wang, Z., Roberts, G., Rudy, D., Krauss-Opatz, B., Kohler, L., Zeidler, H., Schumacher, H. R., Whittum-Hudson, J. A., and Hudson, A. P. (2002) *Chlamydia trachomatis* genes whose products are related to energy metabolism are expressed differentially in active vs. persistent infection. *Microbes. Infect.* **4**, 13–22
22. Iliffe-Lee, E. R., and McClarty, G. (1999) Glucose metabolism in *Chlamydia trachomatis*: the ‘energy parasite’ hypothesis revisited. *Mol. Microbiol.* **33**, 177–187